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The MIM Complex Mediates Preprotein Translocation across the Mitochondrial Inner Membrane and Couples It to the mt-Hsp70/ATP Driving System

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Summary

We have identified a complex in mitochondria that functions as a part of the preprotein import machinery of the inner membrane (MIM complex). Two known components, MIM23 and MIM17, and two novel components, MIM33 and MIM14, were found as constituents of this complex. In the presence of a translocating chain, the outer membrane import machinery (MOM complex) and the MIM complex form translocation contact sites. On the matrix side, the MIM complex is associated with the mt-Hsp70–MIM44 system. We propose a structure of the import machinery in which the MIM complex constitutes a proteinaceous channel that accepts preproteins from the MOM complex, facilitates their reversible transmembrane movement, and mediates unidirectional transport by linkage to the ATP-dependent mt-Hsp70–MIM44 system.

Introduction

Nuclear-encoded mitochondrial preproteins are imported from the cytosol into the matrix in a coordinated fashion across both mitochondrial membranes (Glick et al., 1991; Pfanner et al., 1992). The translocation systems of the outer and inner membranes cooperate in this process (Schleyer and Neupert, 1985; Pfanner et al., 1992; Rassow et al., 1990). The mitochondrial outer membrane contains a protein complex (MOM complex) that recognizes preproteins and facilitates their translocation through a proteinaceous pore or channel (the general insertion pore) (Söllner et al., 1992; Mayer et al., 1995). Matrix-targeted preproteins are then received by the inner membrane import machinery, which can function independently of the outer membrane import apparatus (Segui-Real et al., 1993). In Saccharomyces cerevisiae, three essential proteins of the inner membrane that are required for protein translocation have been identified (Maarse et al., 1992, 1994; Scherer et al., 1992; Dekker et al., 1993; Emtage and Jensen, 1993; Ryan et al., 1994). MIM44 (Isp45) is a hydrophilic protein that is peripherally associated with the inner face of the inner membrane (Maarse et al., 1992; Scherer et al., 1992; Blom et al., 1993). The protein does not contain hydrophobic membrane-spanning segments; however, it was reported to be accessible to protease from the side of the intermembrane space (Maarse et al., 1992). MIM44 recruits mt-Hsp70 to the sites where preproteins enter the matrix space (Kronidou et al., 1994; Rassow et al., 1994; Schneider et al., 1994). The mt-Hsp70–MIM44 complex was proposed to act as a molecular ratchet that facilitates protein translocation in an ATP-dependent manner (Schneider et al., 1994). Unfolded segments of the incoming polypeptide chain appear to interact with the mt-Hsp70–MIM44 complex and stimulate the ATPase of mt-Hsp70. ATP hydrolysis dissociates the mt-Hsp70–MIM44 complex, and mt-Hsp70 is transferred to the incoming polypeptide chain. Mt-Hsp70 in the ADP form binds the preprotein with high affinity and locks the translocation intermediate in the matrix. When binding of mt-Hsp70 is impaired, preproteins slide back in the import channel, and those with less than about 50 amino acid residues exposed to the matrix fall out of the mitochondria (Ungermann et al., 1994).

It was recently discussed that a nucleotide-dependent conformational change of mt-Hsp70 could further facilitate the translocation process by exerting a "pulling force" on the translocating polypeptide chain (Glick, 1995; Pfanner and Meijer, 1995). As ligand-induced conformational changes of a protein of the size of mt-Hsp70 would be considerably small (Vale, 1994), such a translocation motor could assist the unfolding of domains on the outside of the mitochondria at critical stages rather than drive translocation of an entire polypeptide chain.

MIM17 (Sms1) and MIM23 (Mas6) are integral membrane proteins with three to four predicted membrane-spanning segments (Dekker et al., 1993; Emtage and Jensen, 1993; Maarse et al., 1994; Ryan et al., 1994). The two proteins share sequence similarity but are functionally distinct, and they cannot substitute for each other (Maarse et al., 1994; Ryan et al., 1994). Both proteins were found in close proximity to preproteins at early stages of import, when the presequence has not fully traversed the inner membrane (Ryan and Jensen, 1993; Kübrich et al., 1994) and antibodies against MIM23/Mas6 reduced protein import into mitoplasts (Emtage and Jensen, 1993).

Here, we show that MIM23 and MIM17 are part of a complex in the mitochondrial inner membrane (MIM complex), which is required for protein import. In addition, the MIM complex contains two novel proteins, MIM33 and MIM14. When a preprotein spanning both outer and inner membranes is arrested, the MIM complex is organized in contact sites with components of the MOM complex of the outer membrane import apparatus. Our data suggest that the MIM and MOM complexes constitute a channel for protein translocation into the matrix. Individual components of this channel do not interact with preproteins in transit in a tight manner. On the inner face of the inner membrane, the channel is associated with MIM44. This interaction allows for converting the bidirectional sliding of

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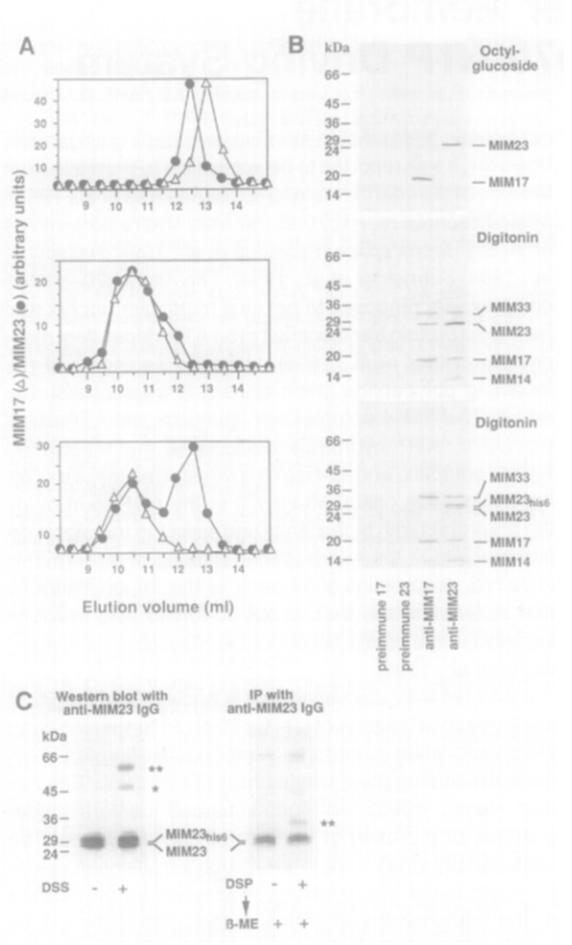

Figure 1. MIM17 and MIM23 Are in a Complex in the Mitochondrial Inner Membrane

(A) Gel filtration analysis of MIM17 and MIM23. Mitochondria from S. cerevisiae strains 334 wild type (top and middle) or from 334 $MIM23_{His6}$ (bottom) were solubilized with either 3.5% octylglucoside (top) or 1% digitonin (middle and bottom) and applied onto a Superose 12 gel filtration column. Fractions were analyzed by SDS–PAGE and Western blotting. The immunoblots were decorated with affinity-purified antibodies against MIM17 and MIM23 and detected using the enhanced chemiluminescence system. MIM17 and MIM23/$MIM23_{His6}$ were quantified by densitometry of X-ray films. Calibration standards eluted at 9.2 ml, thyroglobulin (669 kDa); 11.5 ml, β-amylase (200 kDa); 12 ml, S. cerevisae alcohol dehydrogenase (150 kDa); 13.5 ml, bovine serum albumin (66 kDa); and 15.3 ml, bovine erythrocyte carbonic anhydrase (29 kDa).

(B) Immunoprecipitation of MIM17 and MIM23. Radiolabeled mitochondria (wild type or $MIM23_{His6}$) were solubilized in the presence of 150 mM NaCl with 3.5% octylglucoside and 0.5% digitonin, respectively, and incubated with affinity-purified antibodies against MIM23 or MIM17 prebound to protein A–Sepharose beads. The beads were washed three times for 20 min with the respective solubilization buffer, and the immunoprecipitates were analyzed by SDS–PAGE and fluorography.

(C) Cross-linking of components of the MIM complex. Mitochondria harboring $MIM23_{His6}$ were incubated with or without DSS (200 μM final) for 30 min on ice. Mitochondria were reisolated, and MIM23-specific cross-links of 55 kDa (**) and 45 kDa (*) were detected by SDS–PAGE and Western blotting with anti-MIM23 IgG (left panel). ^{35}S-labeled mitochondria (100 μg/ml) were subjected to cross-linking with DSP (200 μM). A control sample was incubated without DSP. Mitochondria were reisolated and solubilized in the presence of SDS, and immunoprecipitations with anti-MIM23 IgG were performed as specified under Experimental Procedures. The immunoprecipitates were boiled in sample buffer containing β-mercaptoethanol to reduce the DSP cross-links and analyzed by SDS–PAGE and fluorography (right). The 33 kDa band that originates from the 55 kDa adduct with MIM23 is indicated (**).

the unfolded preprotein in the channel into unidirectional movement into the matrix by coupling to the ATP hydrolysis–dependent action of the mt-Hsp70–MIM44 system.

Results

MIM23 and MIM17 Are Part of a High Molecular Weight Complex in the Mitochondrial Inner Membrane

We determined the native molecular masses of MIM23 and MIM17 by gel filtration. When mitochondria were solubilized in n-octyl-β-D-glucopyranoside (octylglucoside), MIM23 eluted from the column in a fraction corresponding to an apparent molecular mass (M_r) of 110 kDa (Figure 1A, top). MIM17 eluted corresponding to an M_r of 90 kDa. Radiolabeled mitochondria were solubilized in octylglucoside, and immunoprecipitations were performed with affinity-purified antibodies. Anti-MIM23 immunoglobulin G (IgG) precipitated a single protein with an electrophoretic mobility on SDS–polyacrylamide gels corresponding to 25 kDa, and anti-MIM17 IgG precipitated a 17 kDa protein (Figure 1B, top). By immunoblotting, the 25 kDa and 17 kDa proteins were confirmed to represent MIM23 and MIM17, respectively (data not shown). When mitochondria were solubilized in digitonin, MIM23 and MIM17 coeluted in a fraction corresponding to an M_r of approximately 280 kDa (Figure 1A, middle). With anti-MIM23 IgG, proteins of 33 kDa, 25 kDa, 17 kDa, and 14 kDa were recovered in the immunoprecipitate (Figure 1B, middle). The same set of proteins was found in immunoprecipitates with anti-MIM17 IgG. This indicates that MIM23 and MIM17 are in a complex in the mitochondrial inner membrane (MIM complex). In addition, the complex apparently contains proteins of 33 kDa and 14 kDa, henceforth referred to as MIM33 and MIM14, respectively. These four proteins form a tight assembly, since they were found in complex at salt concentrations of 50 mM, 150 mM, and 300 mM KCl (data not shown).

A histidine-tagged version of MIM23 ($MIM23_{His6}$) was constructed and expressed in S. cerevisiae under control of the *GAL10* promoter. $MIM23_{His6}$ complemented a disruption of MIM23, indicating that the histidine-tagged protein is functional (data not shown). When $MIM23_{His6}$ was coexpressed with MIM23, the amount of MIM23 plus $MIM23_{His6}$ was about 2.5-fold above the amount of MIM23 in uninduced cells. Mitochondria were prepared and solubilized in digitonin, and the M_r of MIM23 and MIM17 were determined by gel filtration. About 40% of MIM23 and $MIM23_{His6}$ eluted in the high M_r form (Figure 1A, bottom). 60% of the protein eluted corresponding to 110 kDa, i.e., in the same fraction as after solubilization of mitochondria in octylglucoside. The level of MIM17 was not affected by expression of $MIM23_{His6}$. MIM17 coeluted exclusively with the high

M_r form of MIM23/$MIM23_{His6}$. Immunoprecipitations with anti-MIM23 IgG and anti-MIM17 IgG both precipitated MIM23 and $MIM23_{His6}$ as well as MIM33, MIM17, and MIM14 (Figure 1B, bottom). Interestingly, anti-MIM17 IgG precipitated MIM17 and MIM23/$MIM23_{His6}$ in approximately equal amounts, while anti-MIM23 IgG precipitated an approximately 2.5-fold molar excess of MIM23/$MIM23_{His6}$ over MIM17. These observations indicate that $MIM23_{His6}$ assembles into the MIM complex. MIM23/$MIM23_{His6}$ synthesized in excess over other components of the complex remains unassembled in the 110 kDa form.

To characterize the oligomeric state of the 110 kDa form, mitochondria that harbored MIM23 and $MIM23_{His6}$ were solubilized in octylglucoside and applied to Ni–nitrilotriacetic acid agarose (Ni–NTA). $MIM23_{His6}$ was retained on the affinity resin, while MIM23 did not bind (data not shown). Thus, MIM23 and $MIM23_{His6}$ are not in a complex. Accordingly, the 110 kDa form might represent unassembled MIM23 present in a detergent micelle.

To obtain further insight into the composition of the MIM complex, mitochondria that contain $MIM23_{His6}$ were subjected to chemical cross-linking with disuccinimidyl suberate (DSS). A major specific cross-link product with an electrophoretic mobility on SDS–polyacrylamide gels corresponding to 55 kDa was detected with anti-MIM23 IgG by Western blotting. In addition, a minor cross-link product of 45 kDa was seen (Figure 1C, left). Both adducts were also observed when mitochondria were extracted with carbonate (pH 11) prior to cross-linking (data not shown), indicating that MIM23 was cross-linked to integral membrane proteins. The 45 kDa cross-link could represent an adduct of MIM23 with MIM17. However, the MIM17 IgGs were not sensitive enough to detect MIM17-specific cross-links. The 55 kDa major adduct could represent a cross-link between MIM23 and MIM33, but antibodies against MIM33 are not yet available to test this directly. As the 55 kDa cross-link is generated quite efficiently, radiolabeled mitochondria were treated with the cleavable cross-linker dithiobis(propionic acid N-hydroxy)succinimide ester (DSP), which yields the same pattern of MIM23-specific cross-links as DSS (data not shown). Specific adducts with MIM23 were immunoprecipitated with anti-MIM23 IgG. When the DSP cross-links were reduced in the presence of β-mercaptoethanol, a radiolabeled band with the electrophoretic mobility of MIM33 was detected by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and fluorography in addition to MIM23 and $MIM23_{His6}$ (Figure 1C, right). This confirms that MIM33 is associated with the MIM complex. A radiolabeled band corresponding to MIM17 was not detected above background, reflecting the lower efficiency of cross-linking with MIM23.

In summary, these data indicate that MIM23 and MIM17 are in a complex in the inner membrane, termed the MIM complex. In addition to MIM23 and MIM17, the MIM complex contains two novel components, MIM33 and MIM14. These four components can be immunoprecipitated with antibodies against MIM23 as well as with antibodies against MIM17. The coimmunoprecipitation depends on solubilization conditions, which preserve MIM23 and MIM17 in a high molecular weight assembly. MIM33 appears to be an integral membrane protein, and chemical cross-linking provides additional evidence for its association with the MIM complex.

Association of MIM44 with the MIM Complex

We investigated whether MIM44 interacts with the MIM complex. In immunoprecipitates with anti-MIM23 or anti-MIM17 IgG, a radiolabeled band corresponding to MIM44 was not detected above background (see Figure 1). However, the stringency of the immunoprecipitations may have obscured a weaker association of MIM44 with the MIM complex in these experiments. In fact, it turned out that the association of MIM44 with the inner membrane is very labile to salt concentration and detergents. When mitochondria were sonicated in the presence of 100 mM KCl, a significant portion of MIM44 was released from the membrane. At higher salt concentrations, more than 70% of MIM44 were stripped from the membrane (Figure 2A, left). Upon extraction of mitochondria with 0.15% digitonin in the presence of 50 mM NaCl, about 70% of MIM44 was released from the membrane (Figure 2A, right). Under these conditions, the matrix space was opened, but the inner membrane was not solubilized. Solubilization of MIM23, MIM17, and other integral inner membrane proteins required 0.5% digitonin. At higher salt concentrations, the release of MIM44 from the membrane in the presence of 0.15% digitonin was even more efficient (data not shown). Similarly, very low concentrations of Triton X-100 or octylglucoside were sufficient to solubilize MIM44, whereas MIM17 and MIM23 remained membrane associated. To detect a potential interaction of MIM44 with the MIM complex, mitochondria were lysed with 0.5% digitonin in the presence of 50 mM NaCl, and immunoadsorption was performed. The protein A–Sepharose beads containing IgG–antigen complexes were briefly washed, and the immunoprecipitates were dissolved in Laemmli buffer and analyzed by SDS–PAGE and immunoblotting. When mitochondria were solubilized with digitonin, MIM17 and MIM44 were detected in immunoprecipitates with anti-MIM23 IgG (Figure 2B, left). Abundant mitochondrial proteins like the ATP/ADP carrier (AAC) or the β subunit of the F_1-ATPase were not found in the immunoprecipitate (Figure 2B, right). This suggests that MIM44 is specifically associated with the MIM complex. Isp42/MOM38 (Baker et al., 1990; Kiebler et al., 1990) was also not detected in the immunoprecipitate, indicating that the outer membrane import machinery does not interact with the MIM complex in a stable manner. Neither MIM17 nor MIM44 coimmunoprecipitated with MIM23 when mitochondria were solubilized in octylglucoside.

When mitochondria harboring MIM23 and $MIM23_{His6}$ were solubilized at a low salt concentration with digitonin and applied to Ni–NTA, $MIM23_{His6}$ was retained on the resin (Figure 2C). MIM17 was also retained on Ni–NTA, suggesting that the MIM complex remained intact. However, MIM23 without a histidine tag did not bind to Ni–NTA, indicating that it was not associated with $MIM23_{His6}$. Thus, the MIM complex appears to contain a single copy of MIM23. MIM44 was also recovered on Ni–NTA, while neither AAC nor Isp42 were bound to the resin. When a

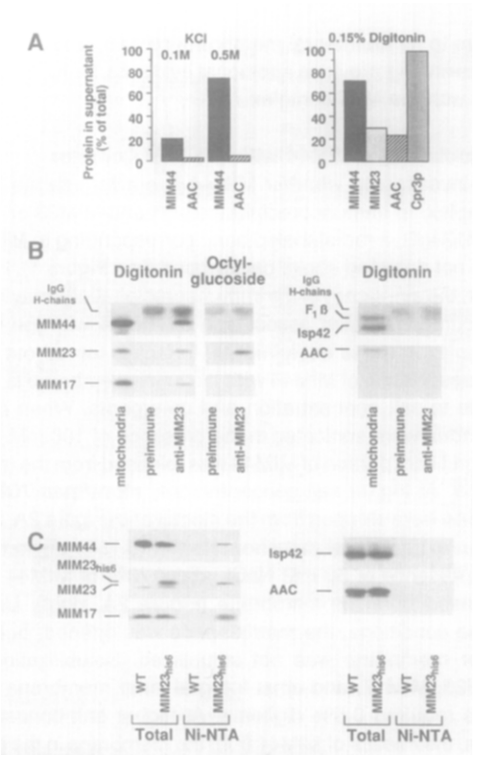

Figure 2. MIM44 Is Associated with the MIM Complex

(A) The association of MIM44 with the inner membranes is sensitive to salt concentration and to detergent. (Left panel) Mitochondria (100 μg/ml) were sonicated and separated into membrane pellet and supernatant in the presence of the indicated concentrations of KCl. The release from the membranes of MIM44 and AAC as a control was analyzed by SDS–PAGE, Western blotting, and densitometry. The amount of protein recovered in supernatant plus pellet was set to 100%. (Right panel) Mitochondria (100 μg) were incubated in 50 mM NaCl, 30 mM HEPES–KOH (pH 7.4), 1 mM PMSF with 0.15% digitonin. Unsolubilized membranes were removed by centrifugation (30 min at 109,000 × g), and proteins in supernatant and pellet were analyzed by SDS–PAGE and Western blotting. The fractions of MIM44 and MIM23 in supernatant and pellet were quantified by densitometry of X-ray films. Markers for inner membrane and matrix space were AAC and mitochondrial cyclophilin (Cpr3p), respectively.

(B) MIM44 can be coimmunoprecipitated with MIM23. Mitochondria (250 μg) were solubilized in the presence of 50 mM NaCl with 0.5% digitonin or 3.5% octylglucoside. The detergent extracts were incubated with affinity-purified anti-MIM23 IgG or with preimmune IgG prebound to protein A–Sepharose beads. The beads were briefly washed (three times for 1 min) with solubilization buffer, and the immunoprecipitates were analyzed by SDS–PAGE and Western blotting. Immunoblots were decorated with antibodies against the indicated antigens.

(C) MIM44 binds in complex with $MIM23_{His6}$ to Ni–NTA agarose. Mitochondria from S. cerevisiae 334 wild type or from 334 $MIM23_{His6}$ were solubilized with 0.5% digitonin and incubated with Ni–NTA agarose beads. Total detergent extracts (Total) and proteins bound to Ni–NTA were analyzed by SDS–PAGE and immunoblotting with antibodies against MIM44, MIM23, MIM17, Isp42, and AAC.

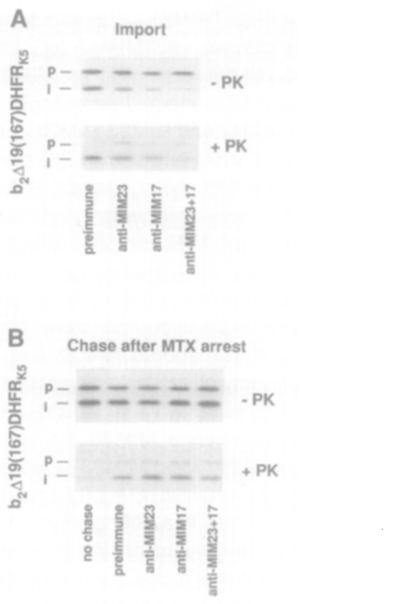

Figure 3. Antibodies against MIM17 and MIM23 Inhibit Preprotein Import at an Early Stage of Translocation across the Inner Membrane

(A) Anti-MIM17 and anti-MIM23 IgGs inhibit import of pb_2Δ19(167)-$DHFR_{K5}$ into mitoplasts. Mitoplasts, generated by swelling of mitochondria in hypotonic buffer solution, were incubated in import buffer with 0.3 mg/ml preimmune IgG or affinity-purified antibodies against MIM17 or MIM23. Radiolabeled pb_2Δ19(167)$DHFR_{K5}$ was added, and import reactions were incubated for 15 min at 25°C. Subsequently, one half of each sample was treated with 0.2 mg/ml proteinase K (PK) for 20 min on ice.

(B) Chase of MTX-arrested b_2Δ19(167)$DHFR_{K5}$ into the matrix is not affected by antibodies against MIM17 and MIM23. Radiolabeled pb_2Δ19(167)$DHFR_{K5}$ was incubated in import buffer with mitoplasts for 15 min at 25°C in the presence of MTX/NADPH. Subsequently, the mitoplasts were reisolated and resuspended in fresh import buffer (50 μl) in the presence of 0.8 mg/ml preimmune serum IgG or affinity-purified anti-MIM17 or anti-MIM23 IgG. The samples were incubated for 1 hr at 25°C. A control sample that received no IgG was left on ice. One half of each sample was then incubated with apyrase and oligomycin for 30 min at 30°C and digested for 20 min at 4°C with 0.2 mg/ml proteinase K. Samples were analyzed by SDS–PAGE and fluorography. Precursor (p) and processed form (i) of b_2Δ19(167)-$DHFR_{K5}$ are indicated.

detergent extract of wild-type mitochondria was applied to Ni–NTA, none of the components of the MIM complex was retained on the resin.

In summary, these observations suggest that the MIM complex contains one copy of MIM23. As MIM23 and the other components in the MIM complex are present in approximately stoichiometric amounts, the complex likely contains a single molecule each of MIM33, MIM23, MIM17, and MIM14. The apparent molecular weight of the MIM complex of approximately 280 kDa is therefore probably due to its presence in a detergent micelle.

MIM44 is associated with the MIM complex. The association is labile in detergent solutions, and only a fraction of MIM44 is recovered with the MIM complex after solubilization of mitochondria. As MIM44, MIM23, and MIM17 are roughly equally abundant in the mitochondria (data not shown), it seems conceivable that MIM44 is an integral part of the MIM complex. On the other hand, the association with the MIM complex could be dynamic, and only a subpopulation of MIM44 might be associated with the complex at a certain time. Isp42 is not found in association with the MIM complex, indicating that in detergent solutions the MIM complex does not interact with the MOM complex of the outer membrane in a stable manner.

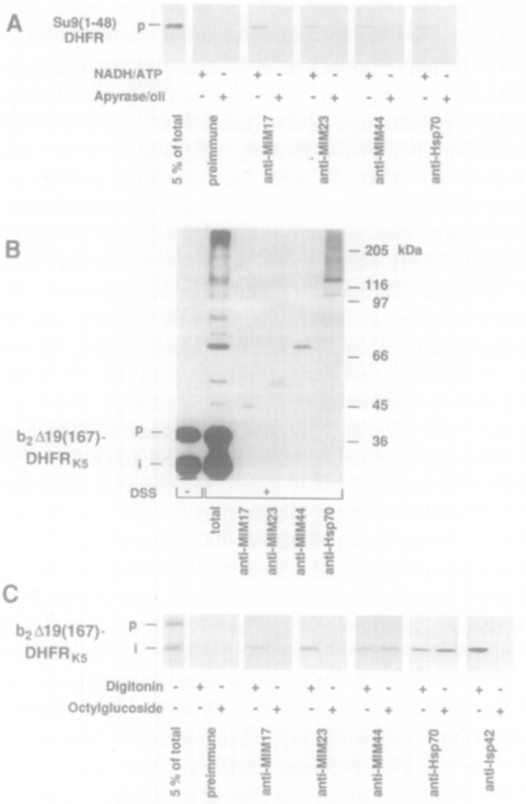

Figure 4. Interaction of the MIM Complex with Preproteins

(A) The association of the presequence of pSu9(1–48)DHFR with the MIM complex requires ATP in the matrix. pSu9(1–48)DHFR was incubated with mitoplasts in the presence of MTX/NADPH to accumulate the preprotein in a membrane-spanning fashion. After import, the mitochondria were incubated with either apyrase/oligomycin or with ATP/NADH. Subsequently, mitochondria were reisolated and solubilized with 0.5% digitonin. After a clarifying spin (1 hr at 109,000 × g), the supernatants were split into five aliquots, which were then subjected to immunoprecipitation with affinity-purified antibodies against MIM17, MIM23, or MIM44, with IgG against mt-Hsp70, or with preimmune IgG.

(B) The MIM complex is in contact with the translocating chain at late stages of protein import. Mitochondria were incubated for 15 min at 30°C with $pb_2\Delta19(167)DHFR_{K5}$ in the presence of MTX/NADPH. Subsequently, the reaction mixture was chilled on ice, and DSS was added to a final concentration of 200 μM. Aliquots were removed before (−DSS) and after cross-linking (+DSS, total). After quenching of the cross-linker, mitochondria were reisolated and solubilized with SDS, Triton X-100. Four aliquots were subjected to immunoprecipitation with affinity-purified antibodies against MIM17, MIM23, or MIM44, or with antiserum against mt-Hsp70. The immunprecipitates were solubilized with Laemmli buffer and analyzed by SDS–PAGE and fluorography.

(C) Membrane-spanning translocation intermediates are coimmunoprecipitated with intact MIM and MOM complexes. $pb_2\Delta19(167)DHFR_{K5}$ was accumulated in a membrane-spanning fashion by incubation with mitochondria in the presence of MTX/NADPH. Subsequently, mitochondria were reisolated and solubilized with either 0.5% digitonin or 3.5% octylglucoside. Six aliquots were then immunoprecipitated with affinity-purified antibodies against MIM17, MIM23, or MIM44, with IgG against mt-Hsp70 or Isp42, or with preimmune IgG. Samples were analyzed by SDS–PAGE and fluorography.

Protein Translocation Mediated by the MIM Complex

Affinity-purified antibodies against MIM23 and MIM17 were used to characterize translocation of preproteins across the inner membrane. In the presence of anti-MIM23 IgG, import of the chimeric precursor $pb_2\Delta19(167)DHFR_{K5}$ (Schneider et al., 1994) into mitoplasts was reduced by approximately 50%, while anti-MIM17 IgG reduced import by 80% (Figure 3A). An increase in the amounts of IgG did not result in further reduction of import, indicating that saturating quantities of the antibodies were added (data not shown). When antibodies against MIM23 and MIM17 were added together, import of $pb_2\Delta19(167)DHFR_{K5}$ was reduced by more than 90%. These observations suggest that portions of the MIM complex that are exposed at the outer face of the inner membrane are required for initiation of protein translocation across the inner membrane.

We investigated whether antibodies against MIM23 and MIM17 affect import at a later stage of protein translocation across the inner membrane. $pb_2\Delta19(167)DHFR_{K5}$ was imported into mitoplasts in the presence of the folate antagonist methotrexate (MTX) that stabilizes the folded dihydrofolate reductase (DHFR) moiety. The fusion protein was processed in the matrix, indicating that it was arrested in a membrane-spanning fashion (Rassow et al., 1989). Subsequently, antibodies were added and then MTX was removed (Gruhler et al., 1995) to allow completion of the import of the translocation intermediate. This chase of MTX-arrested $ib_2\Delta19(167)DHFR_{K5}$ into the matrix was affected neither by anti-MIM23 IgG, nor by anti-MIM17 IgG, nor when both antibodies were present together (Figure 3B).

These observations suggest that domains of MIM17 and MIM23 that are exposed to the intermembrane space have important functions at early steps of protein import before the presequence is translocated across the inner membrane. At later steps, when portions of the translocating chain are already exposed into the matrix, the MIM complex appears to have a passive role.

Interaction of the MIM Complex with Preproteins and with the MOM Complex

To analyze whether the presequence is translocated across the inner membrane in association with the MIM complex, the chimeric precursor pSu9(1–48)DHFR (Ungermann et al., 1994) was imported into mitoplasts in the presence of MTX to arrest the protein in a membrane-spanning fashion. The preprotein was associated with the mitoplasts in a stable manner, and the DHFR moiety was accessible to externally added protease. It was not processed, indicating that the presequence was not fully translocated across the inner membrane. Subsequently, matrix ATP levels were manipulated. Then, the mitochondria were solubilized with digitonin, and immunoprecipitations were performed (Figure 4A). At high levels of matrix ATP, pSu9(1–48)DHFR was immunoprecipitated with antibodies against MIM17, MIM23, MIM44, and mt-Hsp70. This indicates that the MIM complex and the mt-Hsp70–MIM44 complex are associated with the presequence at an early stage of translocation across the inner membrane. When the ATP concentration in the matrix was lowered, the efficiency of these coimmunoprecipitations was significantly reduced. Under these conditions, the mt-Hsp70–MIM44 complex, which energetically drives protein trans-

location, is not formed (Schneider et al., 1994), and pSu9(1–48)DHFR falls out of the mitoplasts and is released into the supernatant (Ungermann et al., 1994). This indicates that the translocating chain is not tightly associated with the MIM complex when the formation of the mt-Hsp70–MIM44 complex is impaired.

We analyzed whether the MIM complex remains in contact with the translocating chain throughout the import process in intact mitochondria. $pb_2\Delta19(167)DHFR_{K5}$ was accumulated in the presence of MTX in a membrane-spanning fashion (Schneider et al., 1994). When DSS was added to the mitochondria, the arrested protein was efficiently cross-linked to MIM17, MIM23, MIM44, and mt-Hsp70 as identified by immunoprecipitations (Figure 4B). Antibodies against mt-Hsp70 precipitated a double band that is typically observed upon cross-linking (Kübrich et al., 1994; Schneider et al., 1994). This indicates that the MIM complex is in contact with the translocating chain at late stages of import after the presequence is cleaved off.

To investigate whether the intactness of the MIM complex is required for the interaction of MIM17 and MIM23 with a preprotein in transit, $pb_2\Delta19(167)DHFR_{K5}$ was arrested with MTX in a membrane-spanning fashion. Subsequently, mitochondria were solubilized with digitonin to preserve the MIM complex or with octylglucoside to disrupt the MIM complex, and immunoprecipitations were performed (Figure 4C). In the presence of digitonin, the processed translocation intermediate ($ib_2\Delta19(167)DHFR_{K5}$) was immunoprecipitated with IgGs against MIM17, MIM23, MIM44, and mt-Hsp70 and also with antibodies against Isp42. The precursor form was not immunoprecipitated. This indicates that $ib_2\Delta19(167)DHFR_{K5}$ was arrested in translocation contact sites between the import machineries of the outer and inner membranes. The inner membrane proteins MIM23 and MIM17 and the outer membrane protein Isp42 are components of such contact sites. When octylglucoside was used for solubilization of mitochondria, $ib_2\Delta19(167)DHFR_{K5}$ was precipitated with antibodies against MIM44 and against mt-Hsp70 but neither with antibodies against MIM23 or MIM17 nor with anti-Isp42 IgG. As the MIM complex disintegrates in the presence of octylglucoside, the intactness of the MIM complex is required for coimmunoprecipitation of a preprotein in transit. This suggests that the individual components MIM23 and MIM17 do not bind to the translocating chain with significant affinity. Similarly, the outer membrane MOM complex is stable in digitonin but disintegrates in octylglucoside (data not shown), suggesting that also the intactness of the MOM complex is required for interaction with preproteins in transit, while Isp42 does not bind to the translocating chain.

In summary, these observations suggest that in translocation contact sites the MIM and MOM complexes form a channel. The components that constitute this channel do not tightly interact with the translocating chain, and $ib_2\Delta19(167)DHFR_{K5}$ is held in this channel by a folded DHFR moiety on one side and by a tightly associated mt-Hsp70 molecule on the other side.

Discussion

Three components of the mitochondrial inner membrane import machinery have been identified by genetic screens: MIM17, MIM23, and MIM44 (Blom et al., 1993; Dekker et al., 1993; Emtage and Jensen, 1993; Maarse et al., 1994; Ryan et al., 1994). We show here that these components are organized in a complex in the inner mitochondrial membrane. We name this complex the MIM complex in analogy to the MOM complex, which facilitates protein translocation across the outer membrane of the mitochondria (Kiebler et al., 1990; Neupert et al., 1990). The MIM complex can be released from the membrane by digitonin as an assembly with an M_r of approximately 280 kDa. It appears to contain one molecule of MIM23 and one of MIM17 and most likely one molecule each of MIM33 and MIM14, novel components whose structures have not yet been determined. MIM44 is associated with the MIM complex in a labile fashion. In detergent solutions, only a fraction of MIM44 is recovered with the MIM complex. MIM44 could be an integral component of the MIM complex, but it might equally well interact with the MIM complex in a dynamic fashion.

Antibodies against MIM23 and MIM17 inhibit protein import into mitoplasts at an early step, namely before the presequence has reached the matrix. Once the preprotein spans the inner membrane, the antibodies no longer affect the translocation process. Portions of the MIM complex that are exposed to the intermembrane space might therefore be involved in the initiation of protein translocation across the inner membrane.

In the presence of a translocating chain, the MIM complex forms translocation contact sites with the import machinery of the outer membrane. In detergent solutions, contact sites are not stable in the absence of a preprotein, yet in the mitochondria, they may exist even without a translocating chain. Arrested translocation intermediates are held in the translocation contact sites mainly by mt-Hsp70 that is bound to the translocating chain on the matrix side. When bound to mt-Hsp70, translocation intermediates targeted to the matrix do not laterally leave the translocation contact sites, provided these are solubilized in detergent solutions that preserve the MIM and MOM complexes. When the MIM and MOM complexes disintegrate, the translocation intermediate remains bound to mt-Hsp70 and MIM44, while MIM23, MIM17, and Isp42 are no longer associated with the preprotein. MIM and MOM complexes might therefore constitute a translocation channel that provides a proteinaceous environment for an unfolded polypeptide chain in transit but does not tightly bind to it. We have recently shown that MIM44 recruits mt-Hsp70 to the sites of protein import (Schneider et al., 1994). The association of MIM44 with the MIM complex might therefore link the passive channel to the molecular machinery that energetically drives the import process.

Interestingly, in the endoplasmic reticulum, preproteins are translocated across the membrane by a proteinaceous channel (Nicchitta et al., 1991; High et al., 1993; Crowley

et al., 1994; Mothes et al., 1994), and BiP, a member of the Hsp70 family, forms a complex with Sec63p, a component of the protein import machinery (Brodsky and Scheckman, 1993). Thus, similarities may exist in the basic organization of the protein import machineries of the endoplasmic reticulum and the mitochondrial inner membrane.

Together with recent findings on the mechanism of presequence translocation across the outer membrane, protein import into the matrix can be subdivided into several distinct steps: within the MOM complex, preproteins are delivered from the outer membrane receptor MOM19/MOM22 (*cis* site) to the general insertion pore. The presequence is translocated across the outer membrane and held at the *trans* site (Mayer et al., 1995). We propose that the outer membrane import machinery that is occupied by a preprotein might screen the inner membrane for a MIM complex, perhaps by lateral diffusion. Then, the presequence is transferred to the MIM complex and inserted into the inner membrane import machinery. The electrical membrane potential $\Delta\Psi$ is required for this insertion, for the transfer of the matrix targeting sequence across the inner membrane, or for both. At the inner face of the inner membrane, the incoming polypeptide chain is taken over by the mt-Hsp70–MIM44 complex (Kronidou et al., 1994; Rassow et al., 1994; Schneider et al., 1994). The mt-Hsp70–MIM44 complex facilitates the translocation of the entire preprotein in an ATP-dependent fashion. Although the detailed mechanism is not entirely understood, the complex appears to act like a molecular ratchet such that the movement of the translocating chain would be driven by Brownian motion and segments of the translocating chain are trapped immediately as they emerge at the inner face of the inner membrane. The MIM and MOM complexes, in the absence of mt-Hsp70, do not tightly interact with preproteins in transit and permit such movements of the translocating chain. Recruitment of mt-Hsp70 to the site of protein import would ensure that segments of the translocating chain are efficiently held in the matrix. A nucleotide-dependent conformational change of mt-Hsp70, MIM44, or both could further facilitate the translocation process by exerting a pulling force on the translocating polypeptide chain to assist unfolding of domains on the outside of the mitochondria at critical stages (Schneider et al., 1994; Glick, 1995; Pfanner and Meijer, 1995).

In summary, early steps in mitochondrial protein import involve translocation of the presequence across the outer membrane by a stepwise transfer to binding sites with increasing affinity and its translocation across the inner membrane by the membrane potential–dependent step. Then, the preprotein is handed over to the mt-Hsp70–MIM44 machinery that drives, in an ATP-dependent manner, the vectorial translocation of the preprotein. At these late stages of import, the MOM and MIM complexes appear to provide a passive channel for the translocating chain.

Experimental Procedures

Subcloning of MIM17 and MIM23 and Generation of Antibodies

MIM23 DNA was amplified from genomic S. cerevisiae DNA by polymerase chain reaction (PCR) using the N-terminal primer 5′-GCC GGA TCC ATG TCG TGG CTT TTT GGA GAT AAG AC-3′ and the C-terminal primer 5′-CGG AAG CTT CAT TTC TCG AGT AGT CTT TTC TTG AC-3′. *MIM17* was amplified using the N-terminal primer 5′-CCC GGA TCC ATG TCA GCC GAT CAT TCG-3′ and the C-terminal primer 5′-CCC AAG CTT AAG CCT GCA GAG GTT GAG AGG-3′. The PCR products were digested with BamHI and HindIII and subcloned into pGem4 and also into pQE13 (Qiagen), yielding the plasmids pGem4–MIM17, pGem4–MIM23, pQE13–MIM17$_{His6}$, and pQE13–MIM23$_{His6}$. The plasmids were transformed in E. coli XL Blue. The double-stranded synthetic oligonucleotide (encoding a histidine tag) 5′-CCG ATA TCT AGA CAT CAC CAT CAC CAT CAC TAG TCG ACA AGC TTG CC-3′ was digested with EcoRV and HindIII and ligated into the HindIII site and the blunt-ended XhoI site of pGem4–MIM23, yielding pGem4–MIM23$_{His6}$. The recombinant histidine-tagged proteins encoded by pQE plasmids were insoluble. They were dissolved in the presence of 7 M urea and purified by Ni–NTA chromatography (Hochuli, 1989). A DNA fragment encoding MIM23 was excised from pGem4–MIM23 with BamHI and HindIII and subcloned into pMAL-cRI (Biolabs) in frame with the gene encoding the maltose-binding protein (MBP). When expressed in E. coli XL blue, the fusion protein MBP–MIM23 was found insoluble in inclusion bodies. Inclusion bodies were purified as described (Arretz et al., 1994). MBP–MIM23 and MIM17$_{His6}$ were used for the generation of antibodies in rabbits. For affinity purification of antibodies, 5 mg of the recombinant proteins MIM17$_{His6}$ and MIM23$_{His6}$, respectively, were coupled to 1 ml of CNBr-activated Sepharose beads (Pharmacia) in 30 mM HEPES–KOH, 2.5% SDS (pH 7.2). 1 ml of anti-MIM17 or anti-MBP–MIM23 serum was diluted with 4 ml of TBS and applied to the respective affinity column. Antibodies were eluted as described (Harlow and Lane, 1988) and dialyzed against water, lyophilized, and dissolved in 50 mM KCl, 30 mM HEPES–KOH (pH 7.2).

Construction of Yep51-MIM23 and Yep51-MIM23$_{His6}$

Yep51 (Broach et al., 1983) was digested with NaeI and SalI, and the ends were filled in with Klenow and religated, yielding Yep51$_{mod}$. A BamHI–HindIII fragment encoding MIM23 was excised from pGem4–MIM23 and subcloned into Yep51$_{mod}$. The cDNA encoding MIM23$_{His6}$ was excised from pGem4–MIM23$_{His6}$ with BamHI and HindIII and subcloned into Yep51$_{mod}$, resulting in Yep51–MIM23$_{His6}$.

Disruption of *MIM23*

A BamHI fragment encoding the *HIS3* gene was ligated into the BglII site of pQE13–MIM23. The disrupted *MIM23* gene was excised with BamHI and PvuII and transformed into the diploid strain MB2-22 (Maarse et al., 1992). His$^+$ cells were subsequently transformed with Yep51–MIM23 or Yep51–MIM23$_{His6}$, and transformants were subjected to random spore analysis (Sherman et al., 1986).

Isolation and Fractionation of Mitochondria

S. cerevisiae 334 was transformed with Yep51–MIM23$_{His6}$ using DMSO-enhanced transformation (Hill et al., 1991). Transformants were grown on selective lactate medium in the presence of 1% galactose, and mitochondria were prepared. Mitochondria from S. cerevisiae strains 334 (wild type) (Hovland et al., 1989), 334Yep51–MIM23$_{His6}$, and D273-10B were prepared as described (Daum et al., 1982). Mitochondria were finally resuspended in HS buffer (20 mM HEPES–KOH, 0.6 M sorbitol [pH 7.2]) at a protein concentration of 10 mg/ml. For generation of mitoplasts, mitochondria were diluted 10-fold with 20 mM HEPES–KOH, 1.0 mg/ml fatty acid–free BSA (pH 7.2) and incubated for 20 min on ice. For salt extraction of peripheral membrane proteins, mitochondria (0.1 mg) were incubated for 1 hr on ice in 1 ml of 30 mM Tris–HCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM o-phenanthroline (pH 8.0), 1 mM PMSF, and KCl as indicated. Every 6 min, the samples were gently sonicated at setting 1 of a Branson sonifier with a 10 s pulse at 30% duty. Membranes were collected by centrifugation as described above, and proteins in the supernatants were precipitated with 6% TCA.

Gel Filtration Analysis

Mitochondria (1 mg of protein) were solubilized with 1% digitonin or 3.5% octylglucoside in 0.2 ml of column buffer (30 mM HEPES–KOH,

200 mM K–acetate, 1.0 mM EDTA, 1.0 mM EGTA [pH 7.4]). The supernatant of a clarifying spin (30 min at 109,000 × g in a Beckman TL100 ultracentrifuge in a TLA45 rotor) was applied on a Superose 12 gel filtration column (25 ml column volume; Pharmacia) and chromatographed at a flow rate of 0.3 ml/min in column buffer containing 1% digitonin or 2% octylglucoside.

Cross-Linking

Mitochondria were diluted to 1 mg/ml in HS, and DSS or DSP (Pierce) was added from a 50-fold stock solution in dimethyl sulfoxide to a final concentration of 200 μM. After incubation for 30 min on ice, the cross-linker was quenched by addition of 1/5 volume of 0.5 M Tris–HCl, 0.6 M sorbitol (pH 8.0). For cross-links with arrested translocation intermediates, ^{35}S-labeled $pb_2\Delta19(167)DHFR_{K5}$ was imported into mitochondria in the presence of MTX/NADPH as described (Rassow et al., 1989). After import, the samples were incubated on ice, and DSS was added as above.

Immunoprecipitations

Immunoprecipitation of MIM17, MIM23, and the MIM complex: ^{35}S-labeled mitochondria were solubilized with either 0.5% (w/v) digitonin or 3.5% octylglucoside in 1 ml of IP buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 2 mM EDTA, 4 mM EGTA, 1 mM PMSF). After a clarifying spin (1 hr at 109,000 × g), the detergent extracts were incubated under gentle shaking for 1 hr at 4°C with affinity-purified anti-MIM17 IgG or anti-MIM23 IgG or the respective preimmune IgG bound to protein A–Sepharose beads. Immunoprecipitates were washed three times for 20 min with 1 ml of IP buffer and analyzed by SDS–PAGE. When radiolabeled mitochondria were used for immunoprecipitations, the protein A–Sepharose beads were preincubated with 0.5 mg/ml unlabeled solubilized mitochondrial protein for 15 min and washed twice with IP buffer before addition of antibodies. Coimmunoprecipitation of MIM44 with the MIM complex: mitochondria (250 μg) were solubilized with 0.5% digitonin or 3.5% octylglucoside in 1 ml of 50 mM NaCl, 30 mM HEPES–KOH (pH 7.4), 1 mM PMSF. After immunoprecipitation with anti-MIM23 IgG or preimmune IgG as above, the protein A–Sepharose beads were washed three times for 1 min with the respective buffer, and the immunoprecipitates were analyzed by SDS–PAGE and Western blotting. Coimmunoprecipitation of MTX-arrested preproteins with components of the import machinery: ^{35}S-labeled fusion proteins $pb_2\Delta19(167)DHFR_{K5}$ or pSu9(1–48)DHFR were imported into mitochondria or mitoplasts in the presence of MTX/NADPH as described (Schneider et al., 1994). When indicated matrix ATP was depleted by incubation of the mitochondria for 20 min at 25°C with apyrase (40 U/ml) and oligomycin (20 μM). After import, the mitochondria were reisolated and washed once in 0.6 M sorbitol, 20 mM HEPES (pH 7.4), 1 mM MTX, 0.8 mM NADPH, 1 mM NADH and solubilized with 0.5% digitonin or 3.5% octylglucoside in 30 mM HEPES–KOH (pH 7.4), 50 mM NaCl, 1 mM PMSF, 2 mM EDTA. After a clarifying spin (1 hr at 109,000 × g), the supernatants were split into aliquots that were then subjected to immunoprecipitation as described above. Immunoprecipitation of cross-links: mitochondria were reisolated and solubilized with 1% SDS in 50 μl of 100 mM Tris–HCl (pH 7.4), and 2 mM PMSF. The samples were kept at 95°C for 3 min before they were diluted 20-fold in TBS, 0.5% Triton X-100. After a clarifying spin (15 min at 15,000 × g), the samples were divided into aliquots that were subjected to immunoprecipitation.

Miscellaneous

Import and MTX arrest of preproteins was performed as published (Rassow et al., 1989). In vivo labeling of cells with [^{35}S]sulfate (Schneider et al., 1994) was with 7.5 mCi/l [^{35}S]sulfate (Amersham) in 2% lactate. Goat anti-rabbit antibodies conjugated to horseradish peroxidase (Sigma) and chemiluminescence kit (ECL, Amersham) were used for immunodecoration.

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References

Arretz, M., Schneider, H., Guiard, B., Brunner, M., and Neupert, W. (1994). Characterization of the mitochondrial processing peptidase of *Neurospora crassa*. J. Biol. Chem. *269*, 4959–4967.

Baker, K., Schaniel, A., Vestweber, D., and Schatz, G. (1990). A yeast mitochondrial outer membrane protein is essential for protein import and cell viability. Nature *348*, 605–609.

Blom, J., Kübrich, M., Rassow, J., Voos, W., Dekker, P. J., Maarse, A. C., Meijer, M., and Pfanner, N. (1993). The essential yeast protein MIM44 (encoded by MPI1) is involved in an early step of preprotein translocation across the mitochondrial inner membrane. Mol. Cell. Biol. *13*, 7364–7371.

Broach, J. R., Li, Y., Wu, L. C., and Jayaram, M. (1983). In Experimental Manipulation of Gene Expression, M. Inouye, ed. (New York: Academic Press), pp. 85–109.

Brodsky, J. L., and Scheckman, R. (1993). A Sec63p–BiP complex from yeast is required for protein translocation in a reconstituted proteoliposome. J. Cell Biol. *123*, 1355–1363.

Crowley, K. S., Liao, S., Worrell, V. E., Reinhart, G. D., and Johnson, A. E. (1994). Secretory proteins move through the endoplasmic reticulum membrane via an aqueous, gated pore. Cell *78*, 461–471.

Daum, G., Gasser, S. M., and Schatz, G. (1982). Import of proteins into mitochondria: energy-dependent, two-step processing of the intermembrane space enzyme cytochrome b_2 by isolated yeast mitochondria. J. Biol. Chem. *257*, 13075–13080.

Dekker, P. J., Keil, P., Rassow, J., Maarse, A. C., Pfanner, N., and Meijer, M. (1993). Identification of MIM23, a putative component of the protein import machinery of the mitochondrial inner membrane. FEBS Lett. *330*, 66–70.

Emtage, J. L., and Jensen, R. E. (1993). MAS6 encodes an essential inner membrane component of the yeast mitochondrial protein import pathway. J. Cell Biol. *122*, 1003–1012.

Glick, B., Wachter, C., and Schatz, G. (1991). Protein import into mitochondria: two systems acting in tandem? Trends Cell Biol. *1*, 99–103.

Glick, B. S. (1995). Can Hsp70 proteins act as force-generating motors? Cell *80*, 11–14.

Gruhler, A., Ono, H., Guiard, B., Neupert, W., and Stuart, R. (1995). A novel intermediate on the import pathway of cytochrome b_2 into mitochondria: evidence for conservative sorting. EMBO J. *14*, 1349–1359.

Harlow, E., and Lane, D. (1988). A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

High, S., Martoglio, B., Görlich, D., Andersen, S. S., Ashford, A. J., Giner, A., Hartman, E., Prehn, S., Rapoport, T. A., and Dobberstein, B. (1993). Site specific photocross-linking reveals that Sec61p and TRAM contact different regions of a membrane inserted signal sequence. J. Biol. Chem. *268*, 26745–26751.

Hill, J., Donald, K. A. I. G., and Griffiths, D. E. (1991). DMSO-enhanced whole cell yeast transformation. Nucl. Acids Res. *19*, 5791.

Hochuli, E. (1989). Aufarbeitung von Bioproteinen: Elegant und wirtschaftlich. Chemische Industrie *12*, 69–70.

Hovland, P., Flick, J., Johnston, M., and Scalafani, R. A. (1989). Galactose as a gratuitous inducer of GAL gene-expression in yeast growing on glucose. Gene *83*, 57–64.

Kiebler, M., Pfaller, R., Söllner, T., Griffiths, G., Horstmann, H., Pfanner, N., and Neupert, W. (1990). Identification of a mitochondrial receptor complex required for recognition and membrane insertion of precursor proteins. Nature *348*, 610–616.

Kronidou, N. G., Opplinger, W., Bolliger, L., Hannavy, K., Glick, B., Schatz, G., and Horst, M. (1994). Dynamic interaction between Isp45 and mitochondrial hsp70 in the protein import system of yeast mito-

chondrial inner membrane. Proc. Natl. Acad. Sci. USA *91*, 12818–12822.

Kübrich, M., Keil, P., Rassow, J., Dekker, P. J., Blom, J., Meijer, M., and Pfanner, N. (1994). The polytopic mitochondrial inner membrane proteins MIM17 and MIM23 operate at the same preprotein import site. FEBS Lett. *349*, 222–228.

Maarse, A. C., Blom, J., Grivell, L. A., and Meijer, M. (1992). MPI1, an essential gene encoding a mitochondrial membrane protein, is possibly involved in protein import into yeast mitochondria. EMBO J. *11*, 3619–3628.

Maarse, A. C., Blom, J., Keil, P., Pfanner, N., and Meijer, M. (1994). Identification of the essential yeast protein MIM17, an integral mitochondrial inner membrane protein involved in protein import. FEBS Lett. *349*, 215–221.

Mayer, A., Neupert, N., and Lill, R. (1995). Mitochondrial protein import: reversible binding of the presequence at the trans side of the outer membrane drives partial translocation and unfolding. Cell *80*, 127–137.

Mothes, W., Prehn, S., and Rapoport, T. A. (1994). Systematic probing of the environment of a translocating secretory protein during translocation through the ER membrane. EMBO J. *13*, 3973–3982.

Neupert, W., Hartl, F.-U., Craig, E. A., and Pfanner, N. (1990). How do polypeptides cross mitochondrial membranes? Cell *63*, 447–450.

Nicchitta, C., Migliaccio, G., and Blobel, G. (1991). Biochemical fractionation and assembly of the membrane components that mediate nascent chain targeting and translocation. Cell *65*, 587–598.

Pfanner, N., and Meijer, M. (1995). Pulling in the proteins. Curr. Biol. *5*, 132–135.

Pfanner, N., Rassow, J., van der Klei, I., and Neupert, W. (1992). A dynamic model of the mitochondrial protein import machinery. Cell *68*, 999–1002.

Rassow, J., Guiard, B., Wienhues, U., Herzog, V., Hartl, F.-U., and Neupert, W. (1989). Translocation arrest by reversible folding of a precursor protein imported into mitochondria: a means to quantitate translocation contact sites. J. Cell Biol. *109*, 1421–1428.

Rassow, J., Hartl, F.-U., Guiard, B., Pfanner, N., and Neupert, W. (1990). Polypetides traverse the mitochondrial envelope in an extended state. FEBS Lett. *275*, 190–194.

Rassow, J., Maarse, A. C., Krainer, E., Kübrich, M., Müller, H., Meijer, M., Craig, E. A., and Pfanner, N. (1994). Mitochondrial protein import: biochemical and genetic evidence for interaction of matrix hsp70 and the inner membrane protein MIM44. J. Cell Biol. *127*, 1547–1556.

Ryan, K. R., and Jensen, R. E. (1993). Mas6p can be cross-linked to an arrested precursor and interacts with other proteins during mitochondrial protein import. J. Biol. Chem. *268*, 23743–23746.

Ryan, K. R., Menold, M. M., Garrett, S., and Jensen, R. E. (1994). SMS1, a high-copy suppressor of the yeast mas6 mutant, encodes an essential inner membrane protein required for mitochondrial protein import. Mol. Biol. Cell *5*, 529–538.

Scherer, P. E., Manning-Krieg, U., Jeno, P., Schatz, G., and Horst, M. (1992). Identification of a 45-kDa protein at the protein import site of the yeast mitochondrial inner membrane. Proc. Natl. Acad. Sci. USA *89*, 11930–11934.

Schleyer, M., and Neupert, W. (1985). Transport of proteins into mitochondria: translocational intermediates spanning contact sites between outer and inner membranes. Cell *43*, 339–350.

Schneider, H. C., Berthold, J., Bauer, M. F., Dietmeier, K., Guiard, B., Brunner, M., and Neupert, W. (1994). Mitochondrial Hsp70/MIM44 complex facilitates protein import. Nature *371*, 768–774.

Segui-Real, B., Kispal, G., Lill, R., and Neupert, W. (1993). Functional independence of the protein translocation machineries in mitochondrial outer and inner membranes: passage of preproteins through the intermembrane space. EMBO J. *12*, 2211–2218.

Sherman, F., Fink, G. R., and Hicks, J. (1986). Methods in Yeast Genetics: A Laboratory Course. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Söllner, T., Rassow, J., Wiedmann, M., Schloßmann, J., Keil, P., Neupert, W., and Pfanner, N. (1992). Mapping of the mitochondrial protein import machinery by stage-specific crosslinking of translocation intermediates. Nature *355*, 84–87.

Ungermann, C., Neupert, W., and Cyr, D. M. (1994). The role of Hsp70 in conferring unidirectionality on protein translocation into mitochondria. Science *266*, 1250–1253.

Vale, R. D. (1994). Getting a grip on myosin. Cell *78*, 733–737.